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14. ABSTRACT

The proposed studies will address the area of emphasis of LCRP to "Understand susceptibility or resistance to treatment". Tumor resistance to chemotherapy is a major cause of treatment failures in lung cancer. To eradicate chemoresistant tumor cells, it is important to identify the subset of tumor cells that can survive from chemotherapy and determine their roles in tumor recurrence. Innovative reporter gene systems are designed to mark quiescent or proliferating lung cancer cells (Aim 1) and then used to track and trace the dynamics of these two populations during the course of chemotherapy (Aim 2). In studies proposed in Aim 3, a killer switch will be introduced to the reporter systems that can enable selective elimination of quiescent or proliferating tumor cells. Using this system, quiescent or proliferating cells will be selectively eliminated to determine their roles in resistance to cytotoxic therapy and subsequent tumor recurrence. The studies will identify and validate the sub-group of lung cancer cells that are responsible for causing treatment failure and disease relapse. In long term, the studies will provide new strategies to eliminate lung cancer resistance toward treatments and to improve the disease-free survival of lung cancer patients, including service members and their family and beneficiaries who suffer from lung cancer.

15. SUBJECT TERMS
Lung cancer, chemoresistance, RBL2, quiescent tumor cells, proliferating tumor cells

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Introduction

Background: Quiescent tumor cells can pose significant challenges to chemo- or radiotherapy that primarily target proliferating cells. This subset of quiescent lung cancer cells, whether they are pre-existing or formed by reprogramming in responses to treatments, can evade cyto-toxic therapy, leading to the persistence of minimal residual disease. Once chemotherapy is withdrawn, these quiescent cells can switch back to their proliferative state and cause tumor recurrence. Recently it is found that in glimoa, a relatively quiescent subset of tumor cells, "with properties similar to those proposed for cancer stem cells, is responsible for sustaining long-term tumor growth through the production of transient populations of highly proliferative cells" after treatment (1). Therefore quiescent cells can be resistant to such anti-proliferative therapy. Interestingly, expression of high levels of quiescence inducing genes has been typically associated with poorer patient survival across multiple cancer types (2). Despite the correlative evidence, not much is known about the exact role of quiescent cancer cells in solid tumor biology, much less their clinical relevance. This pilot study seeks to provide a sound mechanistic insight as to how quiescent lung cancer cells contribute to resistance to chemotherapy, thereby causing disease relapse.

Hypothesis/Rationale/Purpose: Quiescent lung cancer cells, whether they are pre-existing or formed by reprogramming in responses to treatments, can evade cyto-toxic therapy, leading to the persistence of minimal residual disease. Once chemotherapy is withdrawn, these quiescent cells can switch back to their proliferative state and cause tumor recurrence. However, it is a big challenge to track the dynamics of tumor quiescence vs. proliferation during tumor responses toward treatments including chemotherapy and determine the exact roles of quiescent tumor cells in recurrence. Here, innovative reporter gene systems are designed to mark, track, and trace quiescent or proliferating lung cancer cells in vitro and in vivo during chemotherapy. Next a killer switch will be introduced to selectively eliminate quiescent or proliferating tumor cells and determine whether it is quiescent tumor cells that should be targeted to eliminate tumor resistance toward therapy.

Objectives:

(1) To construct and validate novel reporter systems for isolation of quiescent and proliferating lung cancer cells.

(2) To track and trace the quiescent and/or proliferating lung cancer cells during chemotherapy.

(3) To identify the subpopulation of lung cancer cells resistant toward chemotherapy and responsible for tumor recurrence by selective elimination of quiescent or proliferating tumor cells.

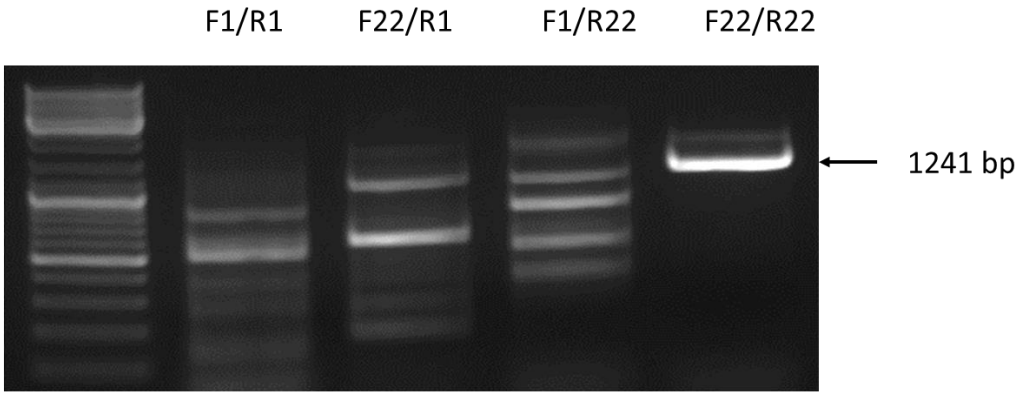
BODY OF REPORT

Scientific portion:

Aim 1: To construct and validate novel reporter systems for isolation of quiescent and proliferating lung cancer cells. (Aim 1).

The biggest challenge to construct reporter systems that reflect cellular quiescence or proliferation is whether the promoter regions cloned can recapitulate its entire promoter activities. RBL2 promoter constructs are not commercially available. We attempted to customer order the RBL2 promoter commercially, but the vendors failed to amplify or clone the RBL2 promoter. Therefore we had to clone the promoter ourselves.

To this end, we used nested PCR approach to amplify the promoter regions of RBL2 and CCND1. As shown in Figure 1, using two sets of primers, we were able to amplify a 1241 bp promoter segment of RBL2 gene.



Primer ID	Primer sequence
proRBL2F1	tgcccaggaagagccccagccat
proRBL2F22	gtgaaaccctgcctgttctga
proRBL2R1	tcttgagcacgggctgctgggaaa
proRBL2R22	agcaattcaaacggcgaagc

Figure 1. Amplification of RBL2 promoter using nested PCR.

Using the exponential megapriming PCR (EMP), we cloned the product into PGL4 vector. For this approach as shown in Figure 2, in the first EMP PCR a forward primer F1 and a reverse primer R1 with overhang exponentially amplify the insert of interest. In the second EMP PCR reaction the purified product is used as a megaprimer to exponentially amplify the target plasmid

together with a forward primer F1 and reverse primer R2 (Ulrich, Andersen, & Schwartz. 2012). Using this approach, we were able to clone the amplified RBL2 promoter into pGL4 promoter (Figure 2, right panel). Further the RBL2 promoter presented cellular activities as shown in **Figure 3**.

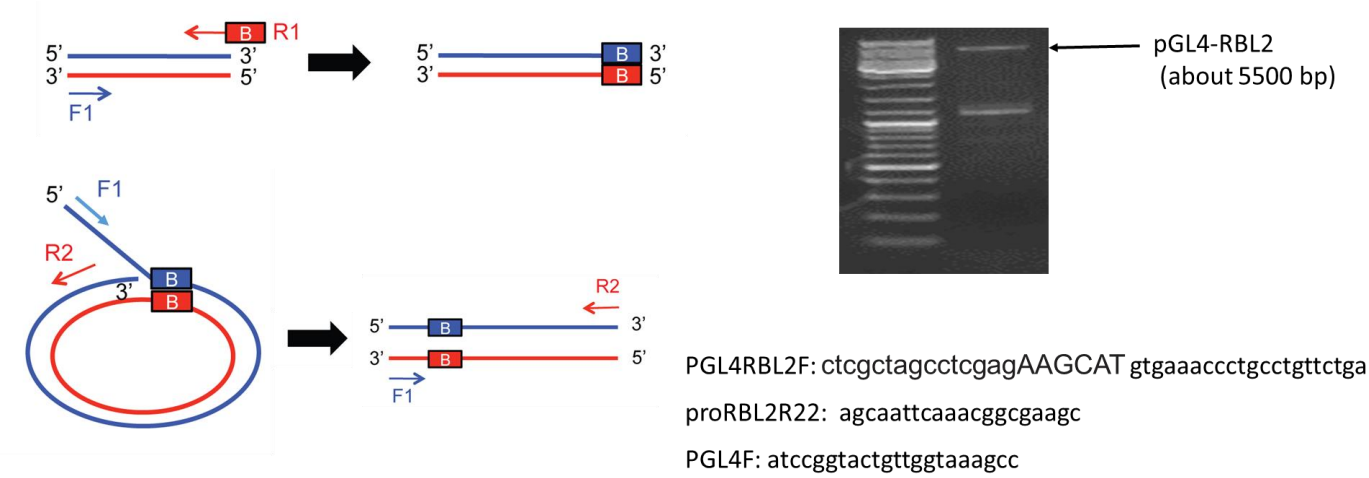


Figure 2. Exponential megapriming cloning of RBL2 promoter into PGL4.

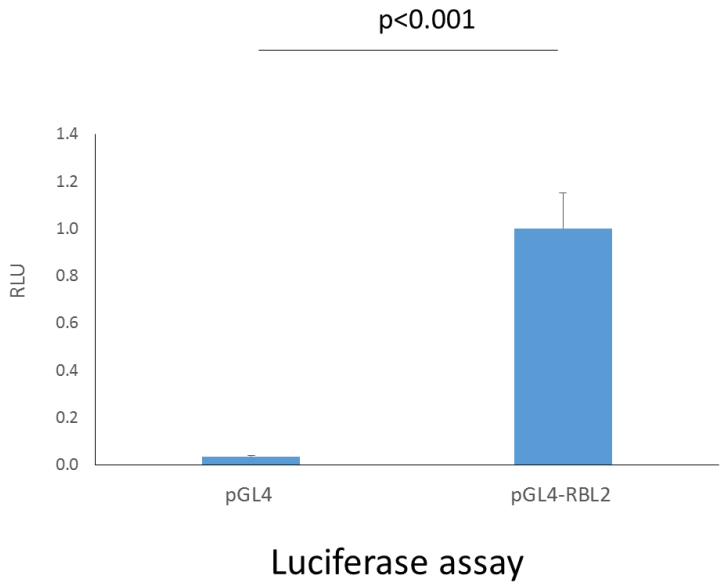
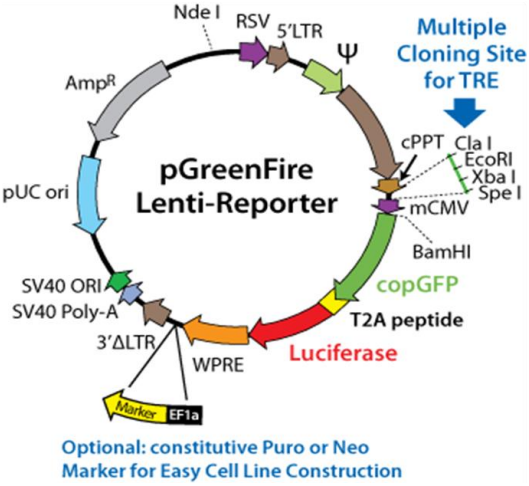


Figure 3. Cellular RBL2 promoter activities.

Next we sub-cloned the RBL2 promoter into pGreenFire vector, in which GFP and luciferase expression is under control of the cloned promoter, to monitor promoter activities in cells and track and trace cells with promoter activities, as shown in **Figure 4**. We were able to clone the RBL2 promoter into pGreenFire vector as confirmed by restriction enzyme digestions (**Figure 5**) and sequencing (not shown).

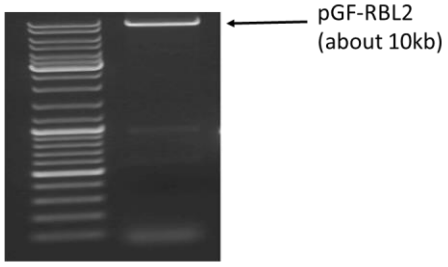
pGreenFire plasmids reconstruction

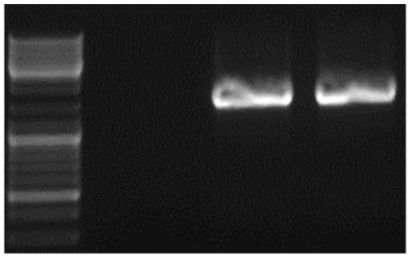


pGreenFire1 (pGF1) is a versatile HIV-based lentivector that co-expresses destabilized copGFP and Firefly Luciferase enabling the detection of both GFP signals as well as Luciferase for quantitative transcription activation reporter assays.

The aim of pGreenFire reconstruction is to insert RBL2 promoter into the multiple cloning site of pGreenFire with EMP cloning method.

Figure 4. pGreenFire vector





EMP PCR

Primers

PGFF: GAATTCTAGAACTAGTTAGGCGTG
RBL2-AC2: GGTACAGTGCAGGGGAAAGAATAGT
gtgaaaccctgcctgttctga
proRBL2R22: agcaattcaaacggcgaagc

Ligation and confirmation

Primers

SeqPGF5.0: GCCCGAAGGAATAGAAGAAGA
SeqPGF3.0: AATGTCGAGAGGCTGGATC

Figure 5. Cloning of RBL2 promoter into pGreenFire (GF) vector.

Aim 2: To track and trace the quiescent and/or proliferating lung cancer cells during chemotherapy (Aim 2).

To determine the dynamics of quiescent / proliferating cells during chemotherapy, first A549 cells were treated with cisplatin (0.2 mg/ml) for 48 hours and RNAs were extracted for analyses of potential changes in expression of genes involved in quiescence.

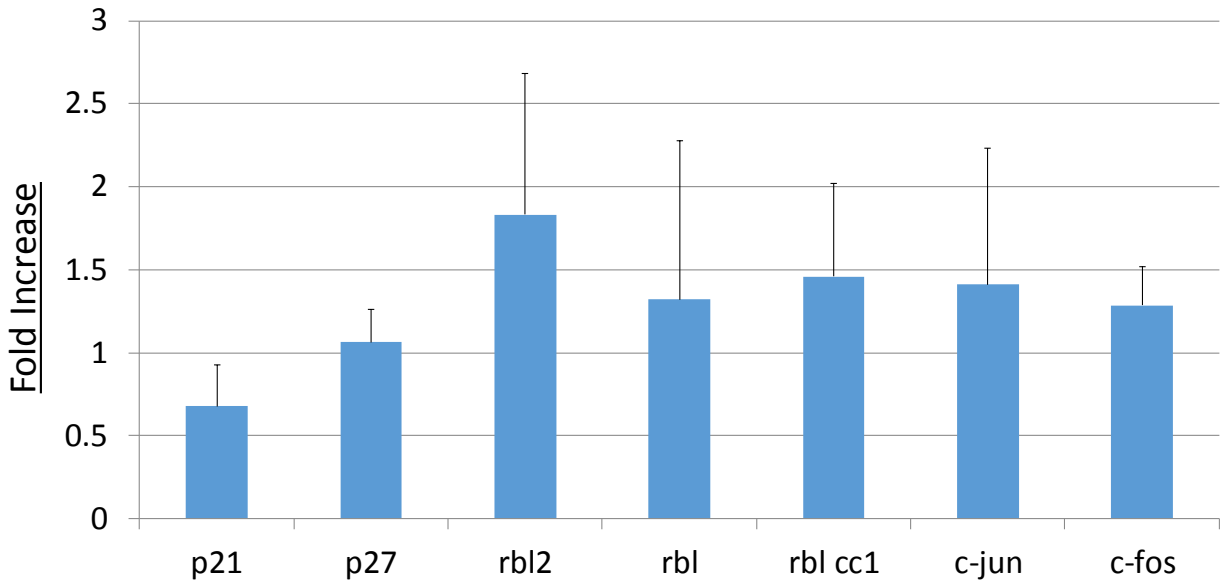


Figure 6. Changes in Expressions of Genes after Cisplatin Treatment

As shown in **Figure 6**, cisplatin treatment did not significantly change in the expression of genes, except that there was about 80% increase in RBL2 RNA level.

Next we determined the dynamics of E2F4 and RBL2, two components of DREAM complex associated with cellular quiescence, in lung cancer cells after chemotherapy. A549 and NCI-H358 cells were treated with cisplatin at different levels for 48 hours. Then the viable cells were fixed and stained for markers of cellular quiescence. LIN-9, a protein associated with RB protein, was also analyzed.

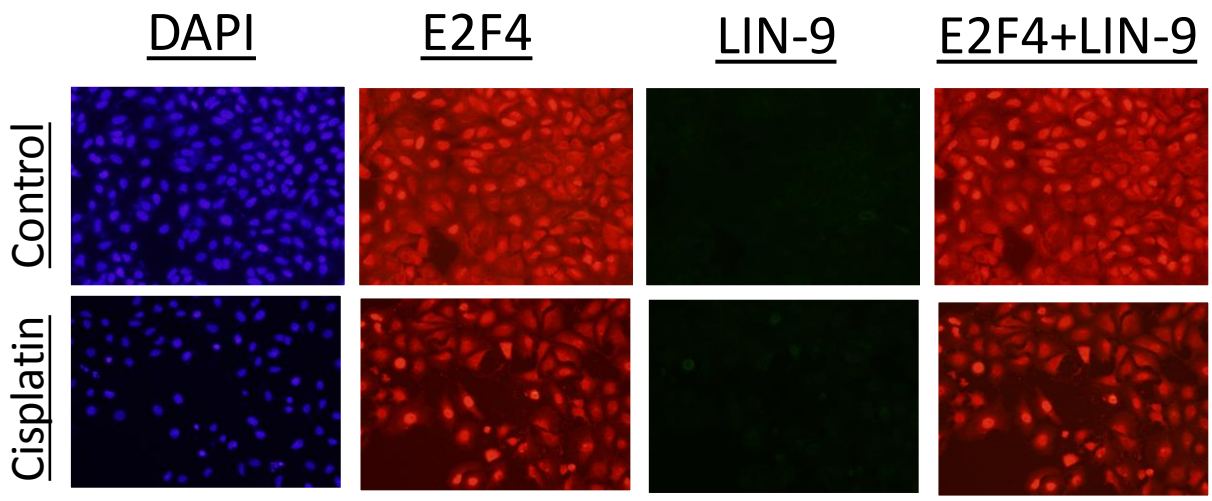


Figure 7. Dynamics of E2F4 and LIN-9 positive A549 cells after cisplatin treatment

As shown in **Figure 7**, in untreated group, some cells were highly positive for E2F4 while in some cells there were low levels of E2F4 staining especially in the nuclei. Cisplatin treatment (0.2 mg/ml for 48 hours) selectively enriched the cells positive for E2F4 in the nuclei. LIN-9 expression was minimally present in A549 cells.

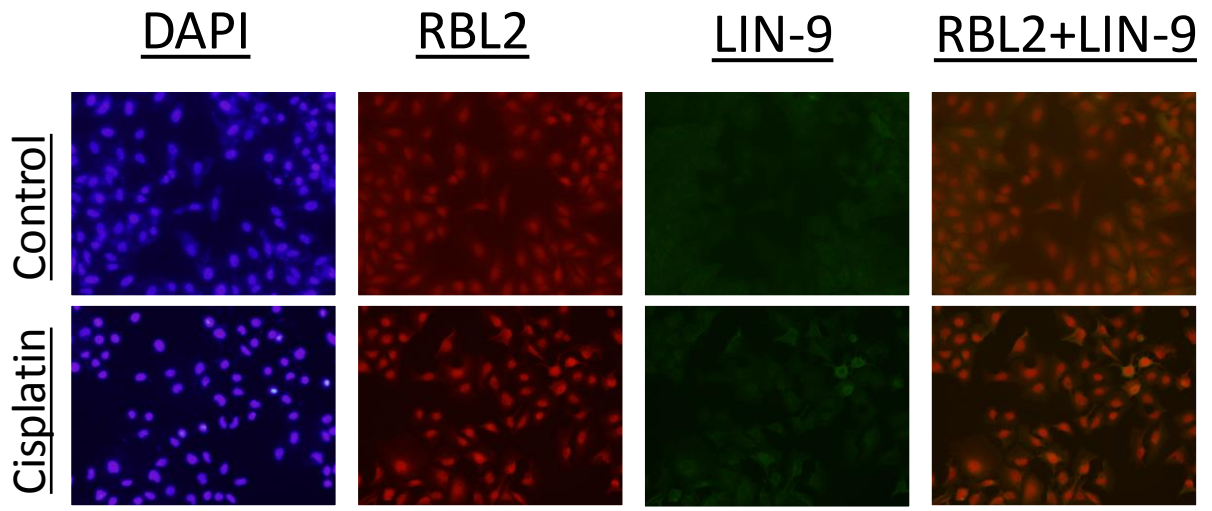


Figure 8. RBL2 and LIN-9 levels in A549 cells after cisplatin treatment

As shown in **Figure 8**, cisplatin treatment (0.2 mg/ml for 48 hours) increased the intensity of RBL2 staining in A549 cells, which is consistent with the increase in RBL2 RNA levels after cisplatin treatment.

Due to the heterogeneity of lung cancer, we examined NCI-H358 cells after cisplatin treatment at different concentrations.

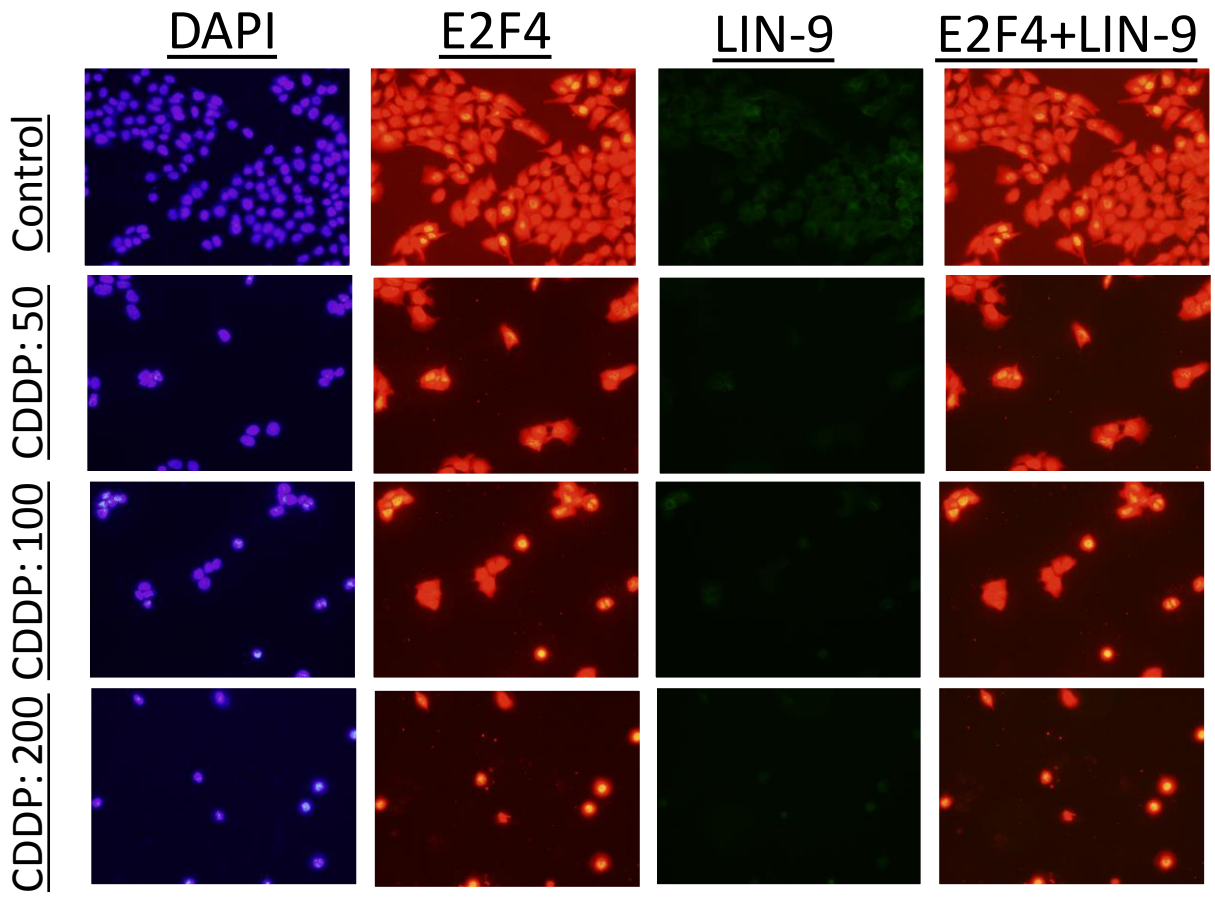


Figure 9 Dynamics of E2F4 and LIN-9 positive NCI-H358 cells after cisplatin treatment

As shown in **Figure 9**, while most NCI-H358 cells were positive for E2F4, some of them were highly positive as indicated by the intensity of staining (yellow color). Interestingly it was these group cells that selectively survived from cisplatin treatment (50 ~ 200 μg/ml for 48 hours).

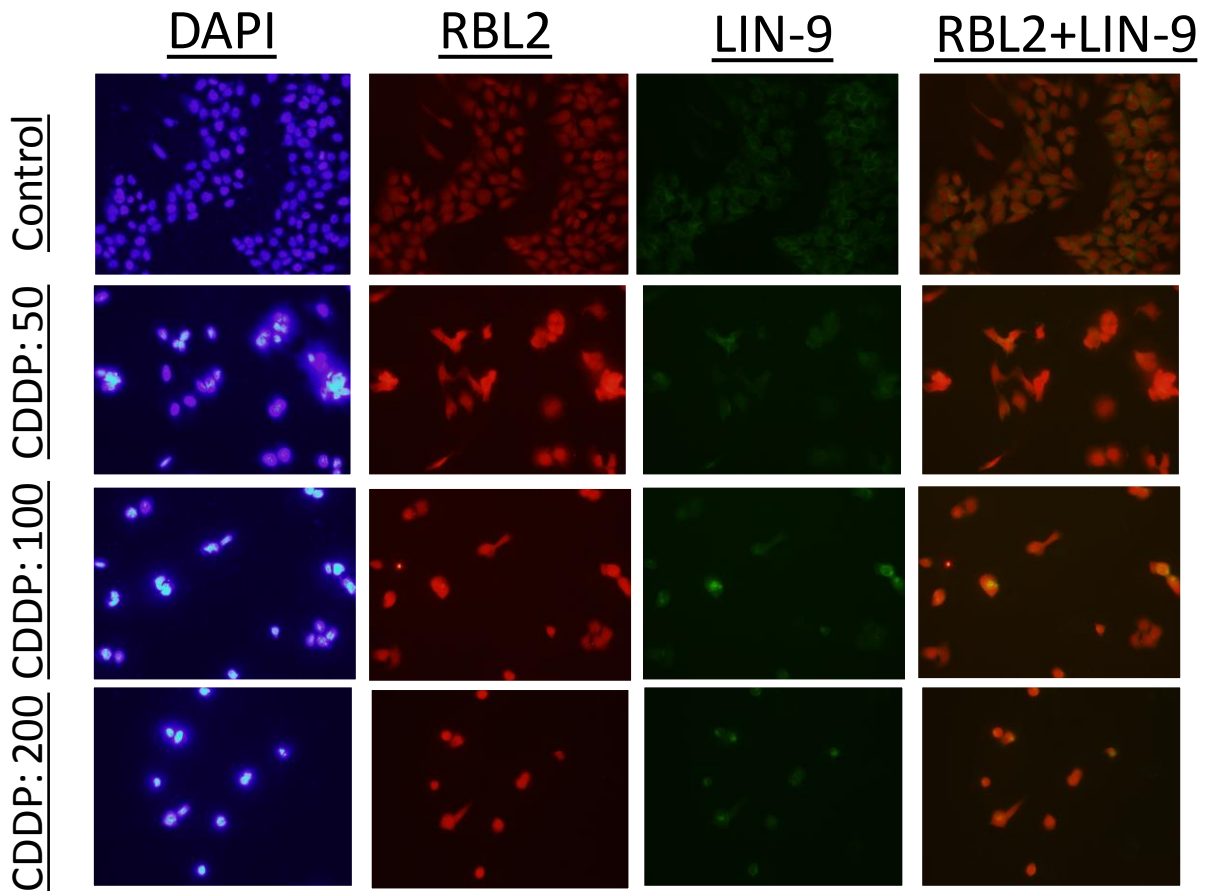


Figure 10. RBL2 and LIN-9 levels in NCI-H358 cells after cisplatin treatment

As shown in **Figure 10**, cisplatin treatment (50 ~ 200 μg/ml) of NCI-H358 cells caused increased positivity of RBL2 staining in the surviving fractions. The results were consistent with what we observed in A549 cells.

Tumor quiescence studies are hampered by the lack of reliable markers. The above studies suggest that RBL expression can be stimulated during chemotherapy, which can confound the interpretation of the data. On the other hand, this stimulation of RBL2 can simply reflect the possibility that quiescence can be induced by stressful conditions such as chemotherapy.

Interestingly we observed that there was a greater heterogeneity of E2F4 positivity in lung cancer cells and cells with strong presence of E2F4 in their nuclei had survival advantages during cisplatin treatment. The observations suggest that E2F4 is a better marker for quiescence. Currently we are exploring the possibility of using E2F4 as another marker for quiescence in lung cancer cells.

Aim 3: To identify the subpopulation of lung cancer cells resistant toward chemotherapy and responsible for tumor recurrence by selective elimination of quiescent or proliferating tumor cells. (Aim 3).

We used RBL2 promoter activities to track and trace quiescent tumor cells during the course of chemotherapy.

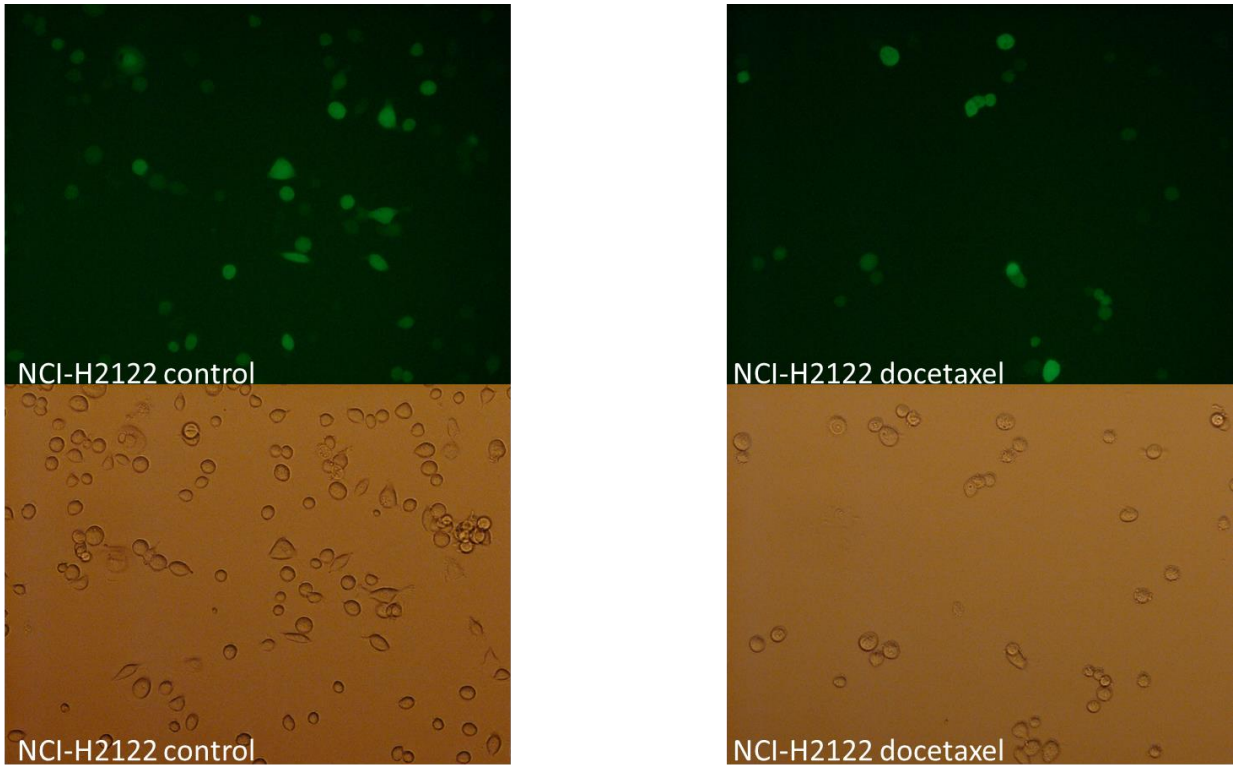


Figure 11. Dynamics of RBL2 promoter active, quiescent tumor cells after docetaxel treatment

Figure 11 depicts the dynamics of quiescent tumor cells in responses to docetaxel treatment: A lot of non-quiescent tumor cells died after docetaxel treatment while significant amount of quiescent tumor cells survived. Enumeration of surviving cells revealed a significant enrichment of RBL2 promoter active, quiescent tumor cells after docetaxel treatment, as shown in **Figure 12 (next page)**. The results suggest that chemotherapy selectively eliminate non-quiescent tumor cells while selectively enrich quiescent tumor cells with active RBL2 promoter activities.

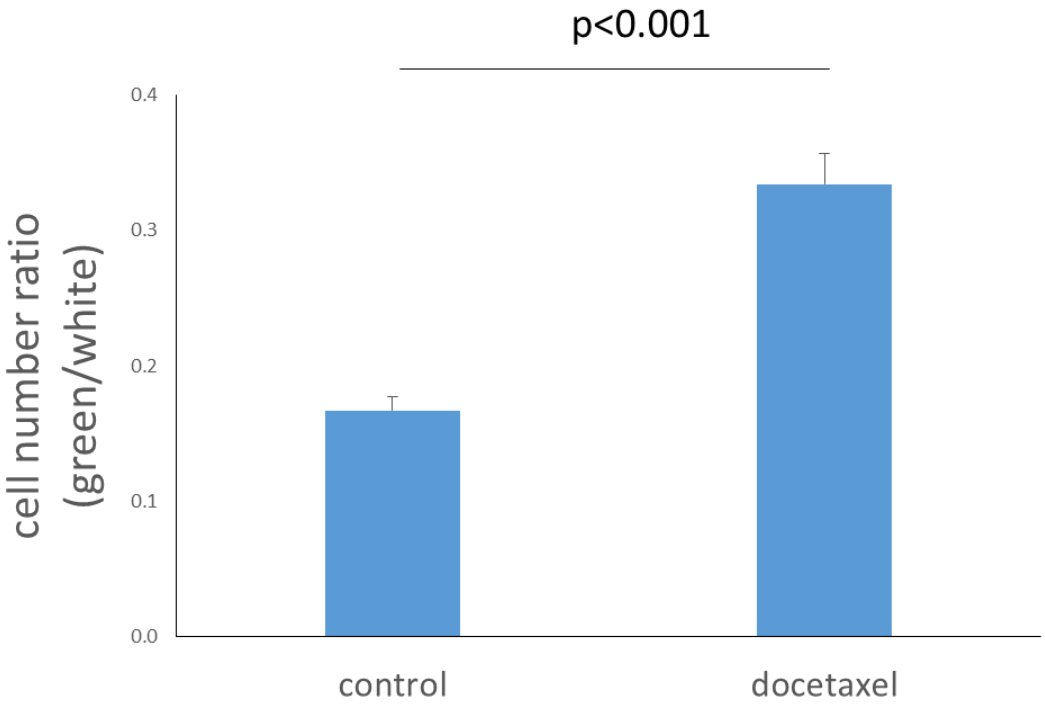


Figure 12. Enrichment of quiescent tumor cells with active RBL2 promoter activities after docetaxel treatment.

KEY RESEARCH ACCOMPLISHMENT and REPORTABLE OUTCOMES

1. We have made several original discoveries regarding the different markers of cellular quiescence and stimulation of RBL2 expression by cisplatin treatment.
2. For the first time we successfully cloned RBL2 promoter, constructed a vector that can be used to mark, track and trace cells with active RBL2 promoter activities.
3. We found that chemotherapy can selectively enrich tumor cells with active RBL2 promoter activities.

Conclusions and significance (So what?):

The studies have found that 1) RBL2 expression can be stimulated in lung cancer cells by cisplatin treatment, 2) Cells with active RBL2 promoter activities have survival advantages during chemotherapy. Future studies are needed to develop approaches to kill those tumor cells with active RBL2 promoter activities to eliminate tumor recurrence after chemotherapy.

APPENDICES

N/A

SUPPORTING DATA

Embedded in the reporting body

REFERENCES

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Ulrich A, Andersen KR, Schwartz TU. Exponential megapriming PCR (EMP) cloning--seamless DNA insertion into any target plasmid without sequence constraints. PLoS One. 2012;7(12):e53360

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